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Target-Cell-Derived tRNA-like Primers for Reverse Transcription Support Retroviral Infection at Low Efficiency

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Reverse transcription of a retroviral genome takes place in the cytoplasm of an infected cell by a process primed by a producer-cell-derived tRNA annealed to an 18-nucleotide primer-binding site (PBS). By an assay involving primer complementation of PBS-mutated vectors we analyzed whether tRNA primers derived from the target cell can sustain reverse transcription during murine leukemia virus (MLV) infection. Transduction efficiencies were 4–5 orders of magnitude below those of comparable producer-cell complementations. However, successful usage of a target-cell-derived tRNA primer was proven by cases of correction of single mismatches between Akv-MLV vectors and complementary tRNA primers toward the primer sequence in the integrated vector. Thus, target-cell-derived tRNA-like primers are able to initiate first-strand cDNA synthesis and plus-strand transfer leading to a complete provirus, suggesting that endogenous tRNAs from the infected cell may also have access to the intracellular viral complex at that step of the replication cycle. © 2002 Elsevier Science (USA)

Key Words: murine leukemia virus; reverse transcription; primer-binding site; tRNA; target-cell complementation.

INTRODUCTION

Retroviruses replicate through reverse transcription of the viral RNA genome into a double-stranded DNA intermediate, which is stably integrated into the host genome as proviral DNA. The overall structure and factor composition of the complex in which reverse transcription occurs are not fully understood.

Retroviral particles are competent to initiate reverse transcription shortly after budding from the producer cell, but there is also evidence that reverse transcription in virions is limited (Lori *et al.,* 1992; Trono, 1992; Arts *et al.,* 1994). Whereas HIV cores are disrupted shortly after virus–cell fusion, Moloney murine leukemia virus (MLV) cores persist longer inside the infected cells (Brown *et al.,* 1987; Grewe *et al.,* 1990; Risco *et al.,* 1995). At that stage MLV cores are not permeable to large macromolecules such as antibodies (Fassati and Goff, 1999), thus probably preventing dilution of viral factors from the reverse transcription complex (RTC) into the cytoplasm. MLV-RTCs that are formed upon viral entry into the cells contain at least the viral genome, CA, IN, RT (Fassati and Goff, 1999), and small RNAs including tRNAs.

The host-encoded cellular tRNA in the retroviral particle serves as a primer for reverse transcription by annealing of its 3′ 18 nucleotides (anti-PBS (Beerens *et al.,* 2001; Gilboa *et al.,* 1979)) to a complementary RNA genome sequence known as the primer-binding site (PBS). Retroviruses differ in their use of tRNA primer and for a given virus the PBS element and its interacting tRNA are highly conserved (Sawyer and Dahlberg, 1973; Peters *et al.,* 1977; Majors and Varmus, 1983; Seiki *et al.,* 1983; Wain-Hobson *et al.,* 1985). However, replication of MLV and MLV-derived vectors is compatible with multiple PBS–tRNA combinations in cell culture (Colicelli and Goff, 1986; Lund *et al.,* 1993, 1997, 2000) and in mice (Lund *et al.,* 1999).

The mechanisms of tRNA recruitment into the virion, tRNA movement within the virion after internalization, and tRNA primer placement before initiation of reverse transcription are not well understood. While genetic analysis based on mutagenesis studies of viral RNA and proteins has already contributed valuable information, investigation of the role of tRNA *in vivo* via mutational analysis is hampered due to its multifunctional nature. However, combinations of PBS-modified vectors and complementary tRNAs have been found to be functional in initiation of retroviral replication in MLV (Lund *et al.,* 1997), HIV (Yu and Morrow, 2000, 2001), and SIVmac239 (Hansen *et al.,* 2001) *in vivo.* In such a "producer-cell" complementation system (Fig. 1) a synthetic tRNA was used to rescue the replication deficiency of a PBS-mutated vector after cotransfection into producer cells and subsequent transduction of target cells with the released virus particles (Lund *et al.,* 1997).

In the present report the producer-cell complementation system (Lund *et al.,* 1997) was modified to elucidate

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the accessibility of the MLV-RTC complex for functional tRNA-like primers derived from the target cells.

We find that the overall transduction efficiency of such a "target-cell complementation" is 4–5 orders of magnitude lower than the comparable producer-cell-derived complementation. However, we provide evidence of rare cases in which target-cell-derived tRNA-like primers are used as functional primers for the initiation of the first-strand cDNA synthesis and plus-strand transfer leading to a complete virus. This finding suggests that cellular tRNAs may also have access to the functional viral complex in the infected cell before initiation of reverse transcription.

RESULTS

Experimental design

We have previously shown that replication of Akv-MLV-derived vectors with a mutationally impaired PBS can be restored by engineered complementary tRNA primers (Fig. 1A), functional in both the initiation of first-strand synthesis and second-strand transfer (Lund *et al.,* 1997; Modin *et al.,* 2000; Hansen *et al.,* 2001). In this setup (producer-cell complementation) vector construct and tRNA primer expressing constructs are cotransfected into the BOSC 23 packaging cell line (Fig. 1B) (Pear *et al.,* 1993). Transduction titers are determined by transfer of transiently produced virus particles to NIH 3T3 cells followed by G418 selection. To provide genetic evidence for tRNA usage, genomic DNA from individual NIH 3T3 colonies can be isolated and analyzed. The PBS region of the integrated provirus derives from a DNA copy of the PBS sequence in the original vector annealed to the anti-PBS region of the tRNA primer at the initiation of reverse transcription (Fig. 2B). Hence, a single marker mutation between vector PBS (PBS-x2m) and the anti-PBS in the tRNA (e.g., $tRNA^{X2Pro}$) results in a mismatched PBS after second-strand transfer. It may be corrected by cellular repair systems according to either template (Fig. 2B) or, if cell division occurs before correction, segregated after DNA replication. Cases of correction toward the newly introduced engineered tRNA thereby would clearly demonstrate the availability and usage of this particular tRNA in the reverse transcription process.

Complementation of a PBS mutant by a tRNA-like primer derived from the target cell (target-cell complementation)

Here we used the potential of this complementation system to elucidate whether the tRNA must be copackaged together with the viral RNA into the virion or, alternatively, whether it may be used for reverse transcription when expressed in the target cell.

$tRNA^{X2pro}$ and $tRNA^{X2Lys3}$, both able to complement the Akv-MLV-derived vector pPBS-x2m (Hansen *et al.,* 2001) in the producer-cell complementation system, were transfected (with a transfection efficiency of approximately 30%) into NIH 3T3 cells to verify expression of the synthetic tRNAs.

Northern analysis, utilizing a probe specific for the last 18 nucleotides of the $tRNA^{X2}$ variants (primer 4), verified transient expression of both tRNAs in NIH 3T3 target cells (Fig. 2A) and the expected size of 75/76 nts for a trimmed and CCA-tailed tRNA. However, $tRNAX2^{Lys3}$ was expressed at higher levels (two- to threefold, Fig. 2A) than $tRNA^{X2Pro}$. As a control for loading and size, the blots were rehybridized with a probe specific for the "anti-PBS" of the endogenous $tRNA^{Pro}$ (primer 5).

Pilot target-cell complementation experiments using the vectors pPBS-x2m-neo and $tRNA^{X2Pro}$/$tRNA^{X2Lys3}$ revealed an impairment of transduction efficiency of 4–5 orders of magnitude compared to the comparable producer-cell complementation experiment, where vector and complementary tRNA were copackaged (5.5×10^4 CFU/ml, Table 1; and Hansen *et al.* (2001)). Most importantly, within the G418-resistant pool of colonies ($<10^1$ CFU/ml, Table 1) cases of PBS correction toward the tRNA sequence were detected (sequence data not shown), strongly suggesting successful usage of the target-cell-derived tRNA.

However, the low efficiency of target-cell complementation makes the analysis sensitive to artifacts, e.g., plasmid contamination. Thus, various control experiments were performed. A new vector, pPBS-x2m-EGFP-IRES-neo, was constructed to discriminate it from vector contamination with pPBS-x2m-neo and pPBS-x2-neo (Fig. 1A). In particular, pPBS-x2-neo contamination (used in previous studies (Lund *et al.,* 1997; Hansen *et al.,* 2001)) could lead to misleading results, since it does not contain the marker mutation compared to the $tRNA^{X2}$ variants and may therefore simulate the usage of the $tRNA^{X2}$ variants in reverse transcription. pPBS-x2m-neo contains the same marker mutation as pBSx2m-EGFP-IRES-neo but only integrated proviruses derived from the new vector will express EGFP after successful complementation, ruling out contaminant-derived PCR amplification from previous experiments.

In accordance with the results obtained for vector pBS-x2m-neo the new vector pPBS-x2m-IRES-EGFP was highly impaired in transduction efficiency ($<10^0$ CFU/ml of supernatant, Table 1). Cotransfection of the cognate primer $tRNA^{X2Pro}$ in the packaging cells restored the replication capacity of the vector to a transfection efficiency level of 1.75×10^4 CFU/ml of supernatant, which is comparable to that of vector pPBS-x2m-neo (5.5×10^4 CFU/ml of supernatant, Table 1).

PCR-based analysis of isolated G418-resistant and EGFP-expressing colonies revealed several cases of usage of the $tRNA^{X2}$ variants when derived from the target cells. In the present experiment (Fig. 2C), 4 of 12 green colonies from NIH 3T3 cells transfected with the

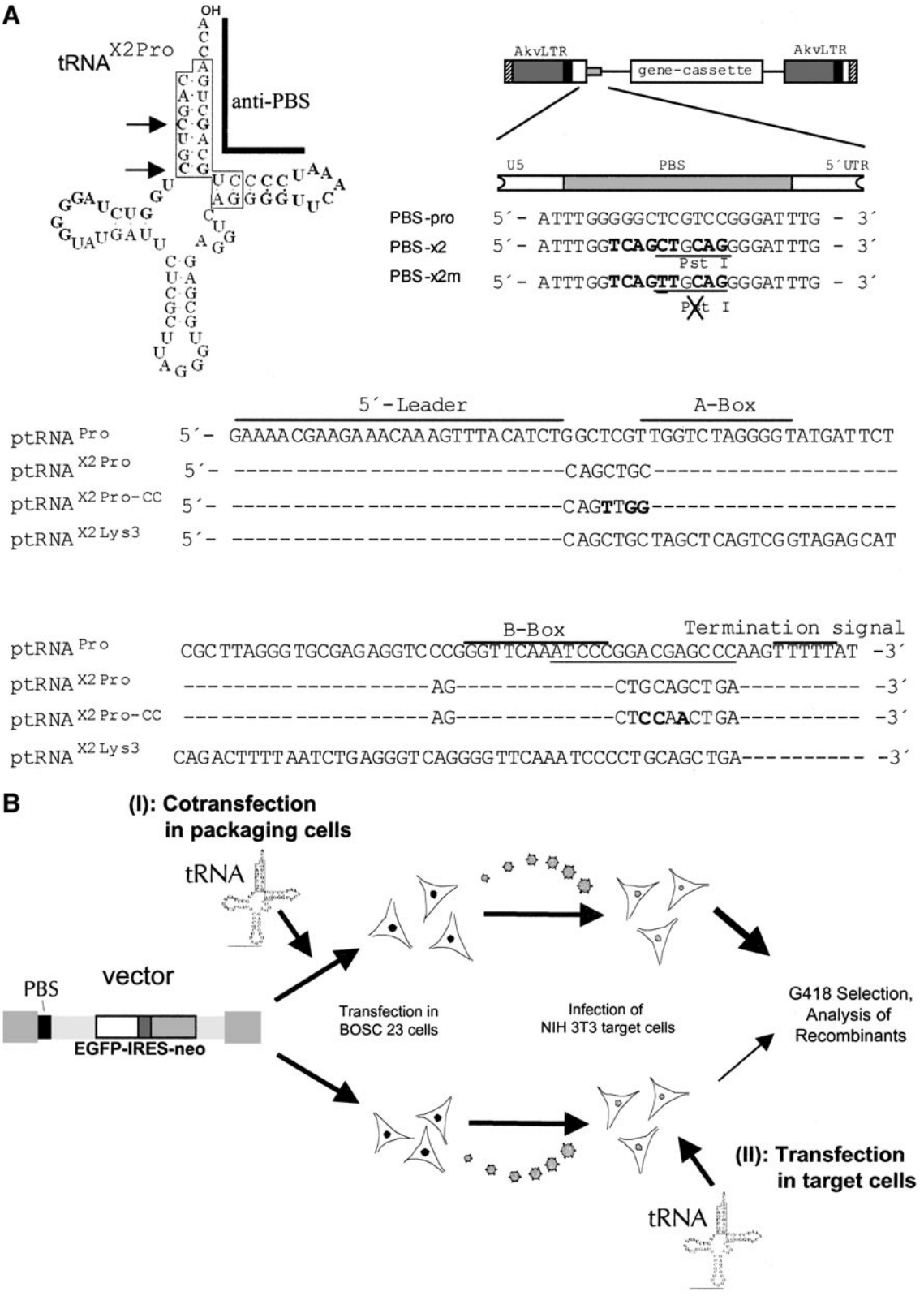


FIG. 1. The complementation system. (A) Structure of vectors and engineered tRNA primers. (Top right) Structure of the Akv-MLV-derived vectors with emphasis on the PBS region. Nucleotide mutations differentiating PBS-Pro and PBS-x2 are shown in boldface type. The single nucleotide difference between PBS-x2 (*Pst*I site) and PBS-x2m (no *Pst*I site) is indicated. (Top left) Putative cloverleaf structure of $tRNA^{X2Pro}$ based on the structure of the murine $tRNA^{Pro}$ (after Harada *et al.,* 1979). Nucleotides mutated compared to $tRNA^{Pro}$ (boxed) and $tRNA^{X2Pro}$/$tRNA^{X2Pro-CC}$ (arrow) are indicated. The anti-PBS region complementary to the PBS-x2 region is indicated (line). (Bottom) Sequences of engineered tRNA molecules. Primary sequence of a 109-bp subfragment of the endogenous murine $tRNA^{Pro}$ gene (Russo *et al.,* 1986) encompassed in synthetic minigenes. Nucleotides mutated in the

tRNA^{X2Pro} variant and 2 of 10 green colonies from transfection with tRNA^{X2Lys3} contained the A → G correction toward the target-cell-transfected tRNA primer sequence. This demonstrates and confirms again successful complementation of the PBS-impaired vector and usage of target-cell-expressed tRNA to initiate reverse transcription.

Four colonies (for tRNA^{X2Pro}) or six colonies (for tRNA^{X2Lys3}) revealed exact identity to the input pPBS-x2m-EGFP-IRES-neo vector. Based on the experimental setup they originated most likely from correction of the mismatch between PBS and tRNA toward the PBS sequence. Less likely they are generated by priming events not involving tRNA/PBS complementarity. Such phenomena of "low-stringency initiation of reverse transcription" within and outside of the PBS region have been described and investigated previously (Mikkelsen *et al.,* 1996, 1998; Lund *et al.,* 2000; Modin *et al.,* 2000) during the analysis of producer-cell complementation experiments using no complementary tRNA (also seen in Table 1). For example, the three clones containing a PBS altered to PBS-pro (Fig. 2C, sequence not shown) derive from low complementary priming with endogenous tRNA^{Pro} as an initiation primer for RT at the PBS site. The one single EGFP-expressing colony from mock-transfected 3T3 cells transduced with pPBS-x2m-EGFP-IRES-neo probably derives from priming outside of the analyzed region, leaving the vector PBS region unaltered during reverse transcription.

The basis of the A/G pair at the critical PBS position of the "mixed" clones (Fig. 2C) was not finally determined, but most likely these colonies contain a mixed population of at least two clones (one corrected, one not corrected).

The genetic evidence for tRNA usage for initiation of RT in the target cells was further confirmed by Southern blot analysis of restriction enzyme-digested genomic DNA of selected clones with PCR-predefined status (Fig. 2C, clones 1–3) at the critical PBS position. As outlined in Fig. 1A, A → G correction creates a *Pst*I site within the PBS region (Fig. 1A) and thereby enables the sequence determination of this position avoiding the more error-prone PCR amplification step. For a PBS-corrected clone toward the tRNA (clone 1), *Pst*I digestion of the 2.24-kb fragment, derived from additional *Pst*I sites, leads to a visible 1.65-kb fragment and an undetected 0.59-kb fragment when hybridizing with an EGFP-specific probe (Fig. 2D). In contrast, clone 3 revealed only a 2.24-kb band and clone 2 reveals both bands (2.24 and 1.65 kb), arguing for the existence of both versions in this colony.

tRNA$^{X2Pro-CC}$ variant of tRNA^{X2Pro} is functional in target-cell complementation

It was shown previously in producer-cell complementation experiments using pPBS-x2-neo that cotransfection with only 1 ng tRNA-expressing plasmid results in transduction titers of about 5–100 CFU per milliliter of supernatant (Hansen *et al.,* 2001). To rule out the possibility that the detected complementation might be based on contamination during the transfection of the packaging cells, a new tRNA^{X2Pro} variant was constructed. It contains a different mismatch mutation (GC → CC) than the vector pPBS-x2m (Fig. 1A).

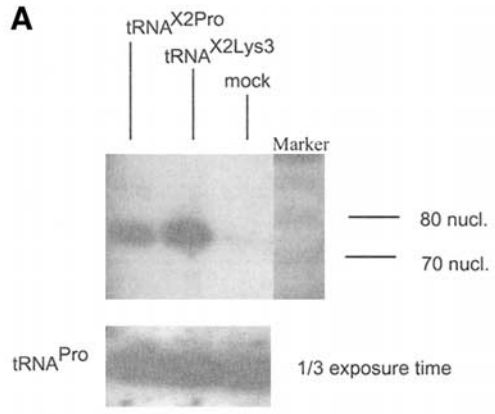
As shown in Fig. 3A, the synthetic tRNA$^{X2Pro-CC}$ primer is expressed in transfected NIH 3T3 cells. Under the membrane washing conditions utilized, the hybridization probe (primer 6) cross-hybridized to some degree to tRNA^{X2Pro} at a position of equal mobility, indicating that tRNA$^{X2Pro-CC}$ is processed similarly to tRNA^{X2Pro} with regard to trimming of the tRNA precursor at the 5′ and 3′ termini. Endogenous snRNPU2 (primer 10) served as a loading control in this experiment (Hansen *et al.,* 2001).

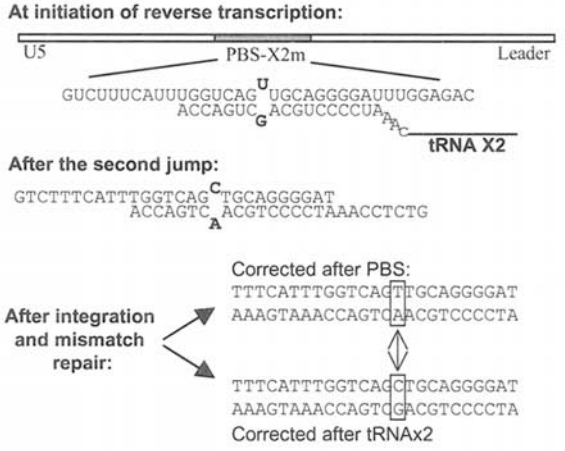
A target-cell complementation experiment using ptRNA$^{X2Pro-CC}$ and vector pPBS-x2m-EGFP-IRES-neo resulted in G418-resistant green colonies at low transduction efficiency ($<0.5 \times 10^1$ CFU per milliliter of supernatant, Table 1). Two clones from >20 clones analyzed contained the GC → CC correction, providing genetic evidence for tRNA$^{X2Pro-CC}$ primer usage to initiate reverse transcription (Fig. 3B, bottom). The remaining clones contained either a vector-like PBS (Fig. 3B, top) or a PBS-pro (not shown) as described for pPBS-x2m-neo complementation (see above).

DISCUSSION

Retrovirus particles contain a significant number of tRNA molecules beside other small RNAs (reviewed in Waters and Mullin (1977)). The enrichment for specific tRNA species in the virion in relation to the pools of available tRNAs (Sawyer and Dahlberg, 1973; Peters *et al.,* 1977; Levin and Seidman, 1981; Mak *et al.,* 1994) suggests this situation to be more advantageous than recruiting a tRNA derived from the target cell after infection. Among other things, the presence of a tRNA annealed to the PBS may allow initiation of reverse tran-

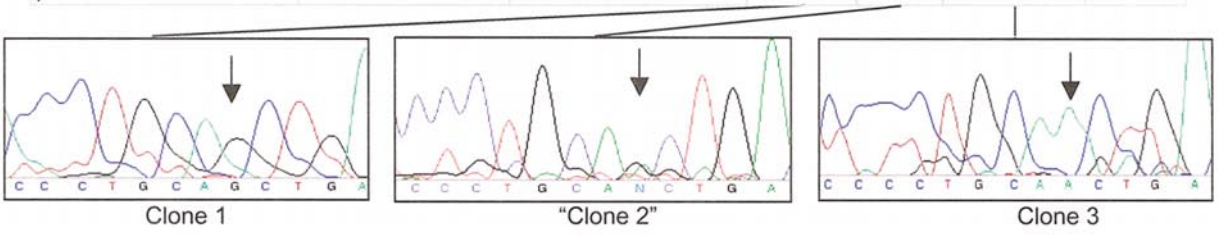
engineered tRNAs tRNA^{X2Pro} (Lund *et al.,* 1997), tRNA^{X2Lys3} (Hansen *et al.,* 2001), and tRNA$^{X2Pro-CC}$ relative to the tRNA^{Pro} gene are indicated. Elements important for transcription are shown above the sequence. Nucleotides annealing to the PBS are underlined. (B) The complementation assay. For producer-cell complementation experiments (I) BOSC 23 producer cells (Pear *et al.,* 1993) are transfected with the PBS-mutated neo-vector in cotransfection with a matching tRNA primer. Subsequently, virion-containing supernatant is used for infection of NIH 3T3 target cells. G418-resistant colonies are counted and analyzed after 10 to 12 days of selection. For target-cell complementation (II) the vector is transfected without any matching tRNA primer; virion-containing supernatant is transferred to NIH 3T3 cells transfected with a tRNA-primer expression plasmid prior to transduction. G418-resistant colonies are isolated, expanded, and analyzed.

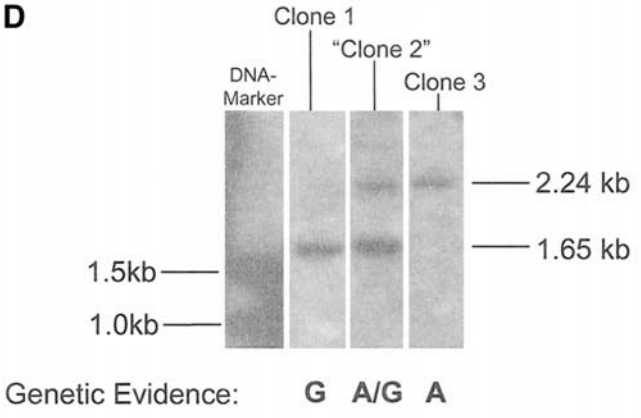




C

transfected vector	transfected tRNA	number of colonies analyzed / total number	sequenced PBS region - mismatch status corrected	mixed	not corrected	Proline
pPBS-x2m-EGFP-IRES-neo	ptRNA X2Pro	12 / 15	4	1	4	3
pPBS-x2m-EGFP-IRES-neo	ptRNA X2Lys3	9 / 10	2	1	6	0
pPBS-x2m-EGFP-IRES-neo	mock	1 / 2	0	0	1	0





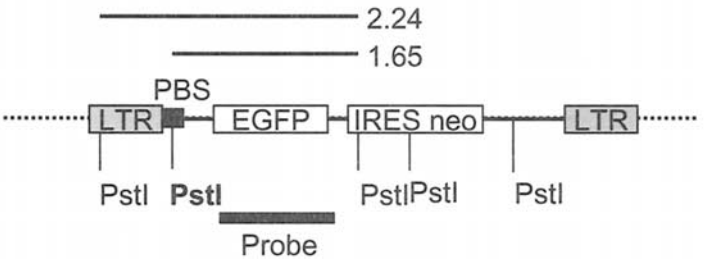


TABLE 1

Vector Transduction Efficiencies in Producer-Cell Complementation and Target-Cell Complementation

Vector construct	Complementary tRNA	Transduction efficiency (CFU/ml)[a]	
		Producer-cell complementation[b]	Target-cell complementation[c]
pPBS-x2m-neo	$tRNA^{X2Pro}$	5.5×10^4	$<10^1$
pPBS-x2m-neo	$tRNA^{X2Lys3}$	$>10^{4d}$	$<10^1$
pPBS-x2m-neo	Mock	$<10^1$	n.d.
pPBS-x2m-EGFP-IRES-neo	$tRNA^{X2Pro}$	1.75×10^4	$<10^1$
pPBS-x2m-EGFP-IRES-neo	Mock	$<10^0$	$<10^0$
Mock	$tRNA^{X2Pro}$	n.d.	0
pPBS-x2m-EGFP-IRES-neo	$tRNA^{X2Pro-CC}$	n.d.	$<0.5 \times 10^1$

[a] Producer-cell complementation experiments and target-cell complementation experiments were performed separately. n.d., not determined.

[b] Producer-cell complementation: NIH/3T3 cells seeded at 10^4 cells/cm^2 were transduced with dilutions of virus particles from BOSC 23 cells transiently transfected with 1 μg of vector + 9 μg of tRNA plasmid.

[c] Target-cell complementation: NIH/3T3 cells were transiently transfected with 4 μg of tRNA plasmid, reseeded at 10^4 cells/cm^2, and transduced with dilutions of virus particles from BOSC 23 cells transiently transfected with 10 μg of vector.

[d] Data from Hansen *et al.* (2001); transfection was performed with 1 μg vector + 2.5 μg tRNA plasmid.

scription shortly after entry. Using two sets of vector/tRNA-primer combinations, we show here that tRNA-like primers from the target cells can initiate reverse transcription only with 4–5 orders of magnitude lower efficiency than tRNA-like primers copackaged in the virion. However, we demonstrate that such tRNA-like primers from the target cells in rare cases are successfully able to initiate reverse transcription. The limited knowledge about the structure of MLV intracellular viral complexes (Fassati and Goff, 1999) as well as mechanisms of normal tRNA incorporation into the viral particle prohibits a detailed discussion of the mechanisms at this stage, but we would like to address the following points:

Endogenous tRNA has been reported to be channeled among components of the translation system and to be associated with proteins at any stage of the translation process (Stapulionis and Deutscher, 1995). Whereas small macromolecules like *Micrococcus* nuclease can enter the functional MLV-RTC complex, larger molecules like antibodies are restricted (Fassati and Goff, 1999). Thus, it is unlikely that tRNAs can enter the RTC when complexed with proteins like ribosomes, elongation factors, and aminoacyl-tRNA synthetases. This may also contribute to the low transduction efficiency seen in our experiments. However, it was not directly proven if the engineered tRNAs are actually complexed with proteins, e.g., in the nucleus, during the transport from the nucleus, or in the cytoplasm. Obviously, however, a subset of tRNAs (maybe the protein-free portion) is able to access the RTC when overexpressed in the target cell.

Posttranscriptional modifications of natural tRNAs may play a role during the formation of the template/primer complex by preventing additional contacts outside of the PBS and allowing the tRNA primer to adopt a stable structure (Fosse *et al.,* 1998). Modifications of single nucleotides in the engineered tRNA primers were not determined in this study but our data show the expressed tRNAs to be processed correctly with regard to

FIG. 2. Target-cell complementation using pPBS-x2m-EGFP-IRES-neo. (A) Expression and processing properties of the engineered $tRNA^{X2}$ variants. (Top) Northern hybridization analysis of expressed engineered $tRNA^{X2}$ variants. Ten μg of total RNA from NIH 3T3 cells expressing the indicated tRNAs after transfection with tRNA minigenes or empty plasmid (mock) was resolved by polyacrylamide gel electrophoresis and hybridized against an oligonucleotide matching the 3′-end of the putative $tRNA^{X2}$ variants. (Bottom) Rehybridization of the same blot with an oligonucleotide probe against endogenous $tRNA^{Pro}$. (B) Genetic evidence—schematic overview. $tRNA^{X2}$ anneals to mismatched PBS-x2m at the initiation of transcription. The marker mutation (arrow) results in a mismatched PBS after second-strand transfer of the reverse transcription process and may be corrected to the PBS template or to the tRNA primer by the cellular DNA-repair machinery prior to integration. The latter case indicates successful tRNA usage and is revealed by sequencing of the PBS region of the integrated provirus. (C) Genetic evidence for tRNAX2-primer usage in target-cell complementation using PCR sequencing. The PBS region from genomic DNA prepared from individual isolated G418-resistant colonies from target-cell complementation experiments was PCR-amplified and sequenced. (Top) Numbers of colonies analyzed in experiments performed with the indicated vector/tRNA-primer combinations. (Bottom) Representative examples (clones 1–3) of the sequenced PBS regions. Mismatched positions are indicated by arrows. (D) Genetic evidence for tRNAX2 primer usage in target-cell complementation using Southern blot analysis. (Top) Genomic DNA from isolated individual G418-resistant colonies (clones 1–3) was *Pst*I digested, resolved by agarose gel electrophoresis, blotted, and hybridized with a probe spanning the EGFP region as outlined in the lower panel. (Bottom) Schematic view of the integrated pBSx2m-EGFP-IRES-neo-derived provirus. The *Pst*I site within the PBS (boldface type) is present only after successful usage of the target-cell-derived tRNA leading to a further digestion of the 2.4-kb *Pst*I–*Pst*I fragment containing the PBS region to a 1.65- and 0.59-kb fragment, respectively.

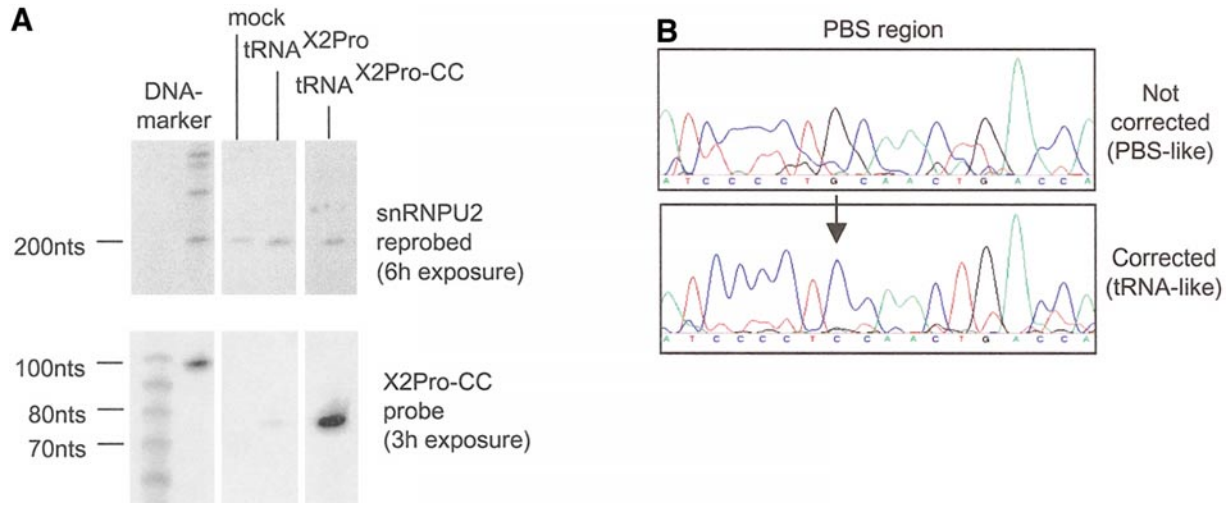


FIG. 3. Target-cell complementation using the modified $tRNA^{X2Pro-CC}$. (A) NIH 3T3 expression and processing properties of the engineered $tRNA^{X2Pro-CC}$. (Bottom) Northern hybridization analysis of expressed engineered $tRNA^{X2}$ variants including $tRNA^{X2Pro-CC}$. Ten μg of total RNA from NIH 3T3 cells expressing the indicated tRNAs after transfection with tRNA minigenes or empty plasmid (mock) was resolved by polyacrylamide gel electrophoresis and hybridized against an oligonucleotide matching the 3′-end of $tRNA^{X2}$ probe matching the 3′-end of $tRNA^{X2Pro-CC}$ including the CCA tail. Reduced-stringency filter-washing conditions result in some cross-hybridization to $tRNA^{X2Pro}$. (Top) Filters were stripped and rehybridized against endogenous U2 small nuclear RNA (U2snRNPU2) as loading control. Exposure times are indicated. (B) Genetic evidence for $tRNA^{X2Pro-CC}$-primer usage in target-cell complementation. The PBS region from genomic DNA prepared from individual isolated G418-resistant colonies from target-cell complementation experiments using $tRNA^{X2Pro-CC}$ was PCR-amplified and sequenced. Top and bottom panels show representative examples of PBS sequences indicating successful usage of the $tRNA^{X2Pro-CC}$ for initiation of reverse transcription (bottom). Mismatch position is indicated (arrow).

trimming of the 5′ and 3′ termini and CCA-tailing. Obviously, some tRNAs contains all necessary modifications to gain access to the RTC and to initiate reverse transcription. However, the possibility cannot be excluded that an artificial modification state might favor usage of the exogenous tRNA primers.

In vitro nucleocapsid (NC) protein has been shown to bind preferentially to single-stranded nucleic acids and to unwind tRNA (Karpel *et al.,* 1987; Khan and Giedroc, 1992; Surovoy *et al.,* 1993) stimulating the annealing of the tRNA primer onto the template and synthesis of minus-strand DNA (Prats *et al.,* 1988; Barat *et al.,* 1989). However, the NC activity critical to load these tRNAs to the PBS is not strictly selective in tRNA binding. Although not directly proven for MLV *in vivo,* it is conceivable that NC is present in the RTC (Gonsky *et al.,* 2001). If the PBS or annealing promoting factors like NC are occupied by other tRNAs (for MLV most likely $tRNA^{Pro}$), target-cell-derived tRNA must be able to disrupt the vRNA–tRNA or tRNA–NC interactions before reverse transcription can be initiated.

It has been shown that different reverse transcriptases recognize specific features provided by the annealed tRNAs (Fosse *et al.,* 1998), but as MLV reverse transcription can be initiated from tRNAs different from the cognate $tRNA^{Pro}$ this activity seems to be nonspecific for a particular tRNA. Furthermore, *pol* products are not required for selection and placement of the tRNA primer onto the PBS in MLV in contrast to ALV, where occupancy of the PBS requires expression of the *pol* gene-coding region (Fu *et al.,* 1997). This relaxed specificity of tRNA usage may also contribute to a successful target-cell tRNA complementation.

In conclusion, we show that tRNA-like primers derived from the target cell can support retroviral infection only at a much lower efficiency than when the tRNA is expressed in the virus producer cells. However, we provide unequivocal evidence for a successful usage of such target-cell-derived tRNA primers for initiation of reverse transcription. This new finding highlights properties of the MLV-RTC complex in terms of tRNA accessibility and tRNA interaction and raises the possibility of the usage of such technology to target specific cells to be competent for viral infection. However, the observed low efficiency does not support its immediate use. Future experiments may address whether the efficiency of infection can be further enhanced. One possibility may be the use of smaller RNA primers (e.g., synthetic RNA oligonucleotides) provided to the target cell before infection.

MATERIALS AND METHODS

Oligonucleotides used in this study 5′ → 3′

The primers used in this study were as follows: Primer 1, CGGAATTCGAAAACGAAGAAACAAAGTTTACATCTCAGTTGGTGGTCTAGGGG; Primer 2, AAAGCTTATAAAAACTTTCAGTTCCAGGGGATTTGAACCCCTG; Primer 3, GGAAAGCTTATAAAAACTTTCAG; Primer 4 (X2 Northern probe), TGGTCAGCTGCAGGGGAAT; Primer 5 (Pro Northern probe), TGGGGGCTCGTCCGGGAT; Primer 6 (X2-Pro-CC Northern probe), TGGTCAGTTGGAGGGGATA; Primer 7,

TCATAAGGCTTAGCCAGCTAACTGCAG; Primer 8, GGCGCCCCTGCGCTGACAGCCGGAACAC; Primer 9, CGCAGGCGCAAAAAGTAGATGC; Primer 10 (snRNP-U2 Northern probe), ATAAGAACAGATACTACACTTGA; Primer 11, CCGGAATTCGAAAACGAAGAAACAAAGTTTACATCTCAGCTGCTGGTCTAGGGGTATGATTCTCGCTTAGGGTGCGAGAGGTCAGGGGTTCAAATCCCCTGCAGCTGAAAGTTTTTAGCTTTCC; Primer 12, GGAAAGCTTAAAAAAGACATGC; Primer 13, CGGAATTCAAAAAGTAAAGCTCTCGTGAAGACAGCTGCTAGCTCAGTCGGTAGAGCATCAGACTTTTAATCTGAGGGTCAGGGGTTCAAATCCCCTGCAGCTGAGCATGTCTTTTTTAAGCTTTCC.

All oligonucleotides were purchased from DNA Technology ApS, Aarhus, Denmark.

Design of vectors and engineered tRNA-like primers

pPBS-x2m-neo was described previously (Lund *et al.,* 1993, 1997). pPBS-x2m-EGFP-IRES-neo was constructed by replacing the *neo* gene (Beck *et al.,* 1982) cassette of pPBS-x2m-neo with a gene cassette containing the EGFP gene (Clontech) and an IRES-neo element (EMCV). The constructs designed to encode the synthetic tRNA-like primer molecules $ptRNA^{X2Pro}$ and $ptRNA^{X2Lys3}$ were constructed from oligonucleotides and cloned essentially as described (Lund *et al.,* 1997; Hansen *et al.,* 2001). $ptRNA^{X2Pro\text{-}CC}$ was generated by standard PCR-mediated mutagenesis (Saiki *et al.,* 1988) using $ptRNA^{X2Pro}$ as template for primer 1 and primer 2 and subcloned in pBluescript.

Cell culture, transfection, and transduction

The human kidney-derived BOSC 23 packaging cell line expressing *gag–pol* and *env* genes (Pear *et al.,* 1993) and NIH 3T3 mouse fibroblast cells were cultured as described (Lund *et al.,* 2000). BOSC 23 were transfected essentially as described (Lund *et al.,* 2000). For producer-cell complementation experiments 1 μg of vector together with 9 μg of tRNA-encoding plasmid was transfected. For target-cell complementation studies, 10 μg of vector alone (or pUC19 mock control) was used. NIH 3T3 transfection was performed using Lipofectamine Plus Reagent (Life Technologies) according to the instructions provided by the manufacturer in 100-mm culture vessels with 4 μg tRNA expression plasmid or pUC19 (mock). Transfection efficiencies were determined with an EGFP-expressing construct (1 μg) and microscopic visualization. Transduction of untransfected NIH 3T3 cells was performed as previously described (Lund *et al.,* 2000). For transduction of transfected NIH 3T3 cells, cells were incubated at 37°C at 5% CO_2 overnight, trypsinized, counted, and reseeded at 1×10^4 cells/ml for transduction with one- to threefold diluted virus-containing supernatant as described (Lund *et al.,* 2000). G418-resistant colonies were isolated and expanded after 10–12 days of selection or expanded as pools.

DNA preparation and Southern hybridization

Trypsinized and pelleted cells from isolated G418-resistant colonies were lysed with DNAzol and genomic DNA was isolated according to the instructions provided by the manufacturer (Molecular Research Center, Inc., Cincinnati, OH).

For Southern hybridization 5–10 μg of genomic DNA was digested by restriction enzyme (10 U per microgram of DNA for 6 h), separated on 1% TBE agarose gel, transferred to Zetaprobe membrane (Bio-Rad), and UV-crosslinked (Stratalinker, Stratagene). A gel-purified DNA probe (EGFP-containing fragment) was labeled by random-priming using Klenow enzyme (La Roche) and [α-^{32}P]dATP and unincorporated label was removed using Microspin G-50 (Pharmacia). Hybridizations and washings were performed at 65°C as described previously (Sorensen *et al.,* 1996).

RNA preparation and Northern hybridization

Total RNA from transiently transfected BOSC 23 or NIH 3T3 cells was extracted with RNA isolator (Genosys) following the instructions provided by the manufacturer. Subsequently the sample was DNase I digested (1 U/μg; 1 h, 37°C), phenol/chloroform extracted, reprecipitated, and dissolved in RNA storage solution (Ambion Inc.). For Northern blot analysis total RNA (5–10 μg) was separated on denaturing 8% polyacrylamide gels and transferred to Zetaprobe membrane (Bio-Rad). Blotting and hybridization conditions were essentially as described in Lund *et al.* (1997) except that hybridization and washing temperatures were set to 52°C for primers 4, 5, and 13. For primer 6 the final washing time was reduced to 5 min. As a control for the amount and integrity of RNA, the filters were stripped (two times 20 min at 90°C in 0.1% SSC and 0.5% SDS) and subsequently hybridized using radiolabeled primer 5 or primer 10.

PCR analysis of transduced vectors (genetic evidence)

Transduced primer-binding site sequences in G418-resistant individual NIH 3T3 colonies were analyzed by PCR. A 1.3-kb fragment (in the case of pPBS-x2m-neo) or a 2.4-kb fragment (in the case of pPBS-x2m-EGFP-IRES-neo) from genomic DNA was amplified using primer 7 (matching Akv-MLV positions 7838–7865) and primer 8 (matching positions 1659–1686 of the *neo* gene (Beck *et al.,* 1982)). PCR amplification parameters were as follows: 1.2 min at 94°C, 1.2 min at 60°C, and 2.5 min at 73°C for 40 cycles. PCR products were gel-purified and directly sequenced on an automated DNA sequencer (ABI 373 DNA sequencer, Applied Biosystems Inc.) using primer 9 matching the Akv-MLV positions 268 to 289.

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